APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1983, p. 1331–1337
0099-2240/83/041331-07$02.00/0

Vol. 45, No. 4

Direct Measurements of Steady-State Kinetics of Cyanobacterial N_2 Uptake by Membrane-Leak Mass Spectrometry and Comparisons Between Nitrogen Fixation and Acetylene Reduction

BENT BORG JENSEN†* AND RAYMOND P. COX
Institute of Biochemistry, Odense University, DK-5230 Odense M, Denmark

Received 24 September 1982/Accepted 29 December 1982

A mass spectrometer with a membrane-covered inlet was used to measure nitrogen fixation by following changes in the concentration of dissolved N_2 in a stirred suspension of the cyanobacterium *Anabaena variabilis* in an open system. The results showed a good fit to Michaelis-Menten kinetics with a K_m for N_2 of 65 μM at 35°C, corresponding to 0.121 atmosphere of N_2. Corresponding values for the K_m for acetylene reduction were 385 μM (0.011 atmosphere at 35°C). Comparison of the values of V_{max} for N_2 uptake with those for the acetylene reduction assay under similar conditions gave an average value of 3.8 for the conversion factor between N_2 and C_2H_2 reduction. Reduction of protons to hydrogen was completely inhibited at sufficiently high concentrations of C_2H_2, but even at saturating N_2 concentrations, 1 mol of H_2 was produced for every mole of N_2 reduced. This explains the finding that the observed C_2H_2/N_2 ratio is higher than the value of 3 expected from the requirement for two electrons for acetylene reduction and six for nitrogen reduction. The results correlate well with a mechanism for N_2 reduction involving the equation: $N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$ which gives a conversion factor between C_2H_2 and N_2 of 4. It is proposed that, in general, 4 is a more appropriate value than 3 for the conversion factor.

Since the first reports by Dilworth (10) and Schöllhorn and Burris (25) of the reduction of acetylene to ethylene by nitrogenase, this reaction has been widely used as an assay of nitrogen fixation. Although many workers who have employed the acetylene reduction technique have expressed their results in terms of N_2 fixation, few have determined the appropriate conversion factor between acetylene reduction and nitrogen fixation. A conversion factor of 3 C_2H_2 reduced per N_2 reduced is often assumed, based on the fact that acetylene reduction requires two electrons and nitrogen reduction requires six electrons. However, the experimentally observed conversion factor has values between 2 and 25 (1).

We have previously reported the use of a mass spectrometer with a membrane-covered inlet to monitor nitrogen fixation by following changes in dissolved N_2 (17). In this paper, we present comparative data on nitrogen fixation and acetylene reduction obtained with a pure culture of *Anabaena variabilis*. We report both K_m and V_{max} for nitrogen fixation and comparable values for acetylene reduction. This allows us to obtain an accurate value for the conversion factor between nitrogen fixation and acetylene reduction.

† Present address: Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, WI 53706.

MATERIALS AND METHODS

Organism and growth conditions. The cyanobacterial strain used in this study was obtained as *Anabaena flos-aquae* CCAP 1403/13a from the Culture Centre of Algae and Protozoa, Cambridge, United Kingdom. It was originally isolated as *A. flos-aquae* MSU A-37 from a sewage oxidation pond by Tischer (29). The strain is the same as *A. flos-aquae* UTEX 1444 and *A. variabilis* ATCC 29413. There is general agreement (2, 12) that the correct nomenclature for the strain is *A. variabilis*, and this name is used here.

The cyanobacteria were cultivated in Allen and Arnon medium as modified by Fay (13) supplemented with 10 mM fructose and buffered with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) adjusted to pH 7.6 with KOH. They were grown at 35°C under continuous illumination (45 μE/m^2 per s) in 100-ml conical flasks containing 50 ml of medium on a rotary shaker (125 rpm). The cyanobacteria were harvested in the exponential growth phase and resuspended in growth medium without fructose. This medium was used for all measurements.

Nitrogen fixation. To measure nitrogen fixation, we used a mass spectrometer to follow the concentration of dissolved N_2 as described previously by Jensen et al. (17). The measuring apparatus resembles that described in detail by Degn et al. (8). It is based on the open system approach developed by Degn and Wohlrab (9). The basic idea behind the open system measurements of nitrogen fixation is that the rate of diffusion (V) of nitrogen across a stable interface between a rapidly stirred liquid phase and a mobile gas phase containing N_2 is directly proportional to the difference between the nitrogen tension of the gas (T_G) and the nitrogen tension in the liquid (T_L). Under steady-state conditions, the rate of diffusion of nitrogen from the gas phase to the liquid phase is equal to the nitrogen consumption by the cyanobacteria; thus, the rate of nitrogen fixation can be calculated from

$$V = K(T_G - T_L)$$

T_L is measured with a mass spectrometer with an inlet covered by a 12.5-μm Teflon membrane through which gases diffuse. We use a quadropole mass spectrometer (type Q8 from VG-Micromass Ltd., Winsford, United Kingdom) fitted with a turbopump (type TPH 100 from A. Pfeiffer Vakumteknik Wetzlar G.m.b.H., Asslar, Federal Republic of Germany). The N_2 concentration was measured by selected ion monitoring of the $m/z = 14$ peak. This peak was selected because CO_2 affects the $m/z = 28$ signal. The instrument allows a rapid scan of up to four different m/z values, allowing simultaneous measurements of four gases.

The value of T_G is given by the signal from the mass spectrometer when the liquid and gas phases are in equilibrium. The dissolved gas concentration corresponding to a particular partial pressure is determined from published values of its solubility (7, 20). The transfer constant K can be determined from the time course of the change in the concentration of dissolved gas following a sudden change in the composition of the gas phase. These transients show an exponential decay to the new equilibrium value with a rate constant equal to K. The value of K is different for different gases and depends on a number of factors, including the temperature, the rate of stirring, the area of the interface, and the sample volume. Degn et al. (8) have provided an extensive discussion of the theoretical background to open system measurements.

The sample chamber was a thermostatted stainless steel vessel with a volume of 7 ml. The sample volume was 4.5 ml and contained the cyanobacterial suspension at a concentration equivalent to 25 to 50 μg of chlorophyll per ml. It was illuminated from below through a Plexiglas window. Saturating light was provided by a slide projector with a yellow filter (Kodak Wratten 16).

The signal from the mass spectrometer was fed into a small laboratory computer, which allowed the experiments to be performed automatically. The dissolved N_2 concentration could be changed by a routine controlling a digital gas mixer (18) through feedback from the mass spectrometer signal. The computer also controlled a shutter to allow illumination of the sample and collected data, allowing the calculation of the rate of light-induced N_2 uptake at different concentrations of dissolved N_2 and hence estimation of K_m and V_{max}. This was done by nonlinear least-squares fit to the Michaelis-Menten equation with intermediate weighting (11).

Acetylene reduction. *Anabaena* suspensions (10 ml containing 2 to 5 μg of chlorophyll per ml) were incubated under saturating illumination at 35°C in 130-ml bottles fitted with serum rubber stoppers. The original gas phase was argon, which was partially replaced with C_2H_2 to the desired final concentration. At appropriate time intervals, 0.5 ml of the gas phase was withdrawn, and the concentration of ethylene was determined by gas chromatography with a PYE 104 gas chromatograph (W. G. Pye and Co. Ltd., York Street, Cambridge, United Kingdom) fitted with a flame ionization detector and a Porapak R column. The carrier gas was N_2, with a flow rate of 20 ml/min. The oven temperature was 50°C.

Chlorophyll estimations. Samples (1 ml each) were extracted in 9 ml of 100% methanol. The concentration of chlorophyll *a* was determined from the absorbance at 665 nm with the extinction coefficient reported by Mackinney (19).

RESULTS

Mass spectrometric measurements of N_2 uptake. The use of the mass spectrometer to measure N_2 uptake by illuminated cyanobacteria has been reported previously (17). A typical series of traces from the mass spectrometer after modification of the apparatus to allow computer control of the experiment is shown in Fig. 1.

At the start of the experiment, the cyanobacteria were in the dark. The composition of the gas phase was 15% N_2 and 85% Ar. The N_2 trace was the same as that observed in the absence of cyanobacteria and corresponds to the concentration in equilibrium with the gas phase. Illumination caused the N_2 concentration to move to a new steady-state level. The difference between the light and dark steady-state levels can be multiplied by the gas transfer constant K to give the rate of light-induced nitrogen fixation as described in the equation given above. The shutter was then closed, and the N_2 concentration was changed to a new value by the computer with the feedback routine. The cyanobacteria were illuminated again once the dissolved N_2 concentration had stabilized to a new value.

In some initial experiments, we checked that at a constant concentration of dissolved N_2, the rate of N_2 uptake during illumination remained stable for several hours, even in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and/or in the absence of CO_2.

The experiment was performed with a scanning mass spectrometer, which allows us to monitor several gases simultaneously. Measurements of O_2 ($m/z = 32$) and H_2 ($m/z = 2$) are also shown in Fig. 1. In the dark, the concentrations of O_2 and H_2 were, as expected, indistinguishable from zero. At the start of the illumination, the H_2 trace increased as the result of light-induced H_2 production by the cyanobacteria,

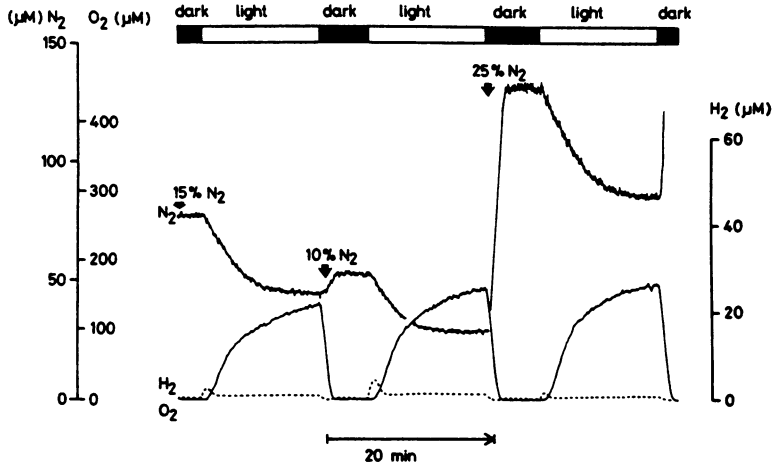

FIG. 1. Changes in dissolved N_2, O_2, and H_2 in a suspension of *A. variabilis*. The cyanobacteria were resuspended at a concentration corresponding to 50 μg of chlorophyll per ml. Measurements were made at 35°C. The gas phase contained argon and different concentrations of N_2 as indicated. N_2 was measured at $m/z = 14$, O_2 at $m/z = 32$, and H_2 at $m/z = 2$.

but as soon as the O_2 concentration in the medium increased due to photosynthetic oxygen evolution, the H_2 trace fell to almost zero and stayed there throughout the illumination period. This is because, in the presence of O_2, the cyanobacteria reoxidize the H_2 produced by nitrogenase. As we have shown previously (17), the H_2 trace attains a steady-state level, indicating a constant rate of H_2 production, if DCMU is added to inhibit photosynthetic oxygen evolution.

The O_2 trace increases after a short lag phase and attains a steady-state level, indicating a constant rate of photosynthetic oxygen evolution, when the cyanobacteria are illuminated. Although the experiment was carried out with a gas phase without CO_2, the rate of photosynthetic oxygen evolution was constant throughout the assay period. The addition of CO_2 or HCO_3^- to the sample increased the cyanobacterial oxygen evolution (not shown). In some experiments, we also measured CO_2 at $m/z = 44$, but the CO_2 trace was indistinguishable from zero throughout the experiment (data not shown).

We assume that the net production of O_2 during illumination is the result of the photosynthetic refixation of CO_2 produced during the breakdown of storage carbohydrates to provide one or more of the requirements for nitrogen fixation: reductant, carbon skeletons, and ATP.

Steady-state kinetics of N_2 uptake. Figure 2 shows the rate of light-induced N_2 uptake as a function of the steady-state N_2 concentration in the light, obtained from a series of experiments similar to that shown in Fig. 1. The curve through the points corresponds to the calculated best fit to the Michaelis-Menten equation, and the experimental points show an excellent fit to the theoretical curve.

Values for K_m and V_{max} for N_2 uptake from five independent experiments with different batches of *A. variabilis* are shown in Table 1. The V_{max} values vary between 16 and 32 μmol of N_2 per h per mg of chlorophyll, with a mean of

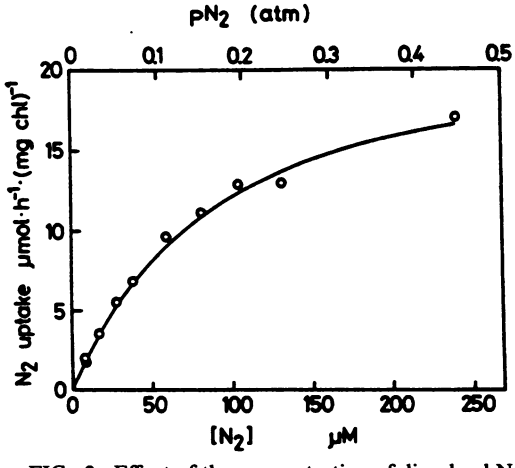

FIG. 2. Effect of the concentration of dissolved N_2 on the rate of N_2 uptake by *A. variabilis*. Rates of light-induced N_2 uptake were calculated by using the equation given in the text from a series of experiments similar to that shown in Fig. 1. T_G corresponds to the dissolved N_2 concentration measured in the dark and T_L to that measured after the steady-state had been reached in the light. The line through the experimental points corresponds to the best fit to the Michaelis-Menten equation. Other experiment details were as described in the text and the legend to Fig. 1.

TABLE 1. Steady-state kinetic parameters for light-induced N_2 uptake by *A. variabilis*

Batch	V_{max} (μmol of N_2 per h per mg of chlorophyll)	K_m	
		μM	kPa
1	20	71	13
2	24	54	10
3	16	62	12
4	32	62	12
5	24	95	18
Mean ± SD	23 ± 6	69 ± 16	13 ± 3

23; K_m values vary between 54 and 95 μM, with a mean of 69.

Steady-state kinetics of acetylene reduction. To allow a direct comparison with our results on N_2 uptake, we measured the activity of the cyanobacteria under similar conditions by using the conventional acetylene reduction assay. Figure 3 shows the effect of acetylene concentration on the rate of ethylene production, which showed a lag phase of about 5 min and was then linear throughout the assay period (30 min). The experimental points again show an excellent fit to the theoretical curve for the Michaelis-Menten equation.

The average value of K_m for light-induced acetylene reduction at 35°C for five different batches of *A. variabilis* was 385 ± 50 μM (standard deviation), corresponding to a partial pressure of 1.1 ± 0.1 kPa. The average value of V_{max} was 82 ± 17 μmol of C_2H_2 per h per mg of chlorophyll. The difference between the K_m values at 35°C for acetylene reduction (385 μM) and N_2 fixation (65 μM) indicates that, on a molar basis, the affinity of the enzyme for nitrogen is greater than the affinity for acetylene. The solubility of acetylene in water is 35.8 mM at 1 atmosphere of C_2H_2 and 35°C (20), or about 60 times greater than the solubility of N_2, which is 0.6 mM at 1 atmosphere of N_2 and 35°C (7). However, as a result of the greater solubility of acetylene in water, C_2H_2 reduction is saturated at a much smaller partial pressure than N_2 fixation.

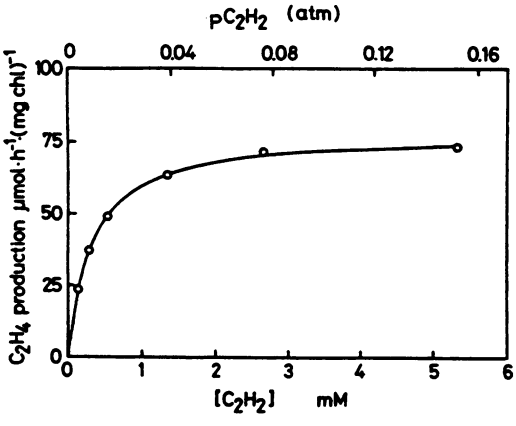

FIG. 3. Effect of the concentration of dissolved C_2H_2 on C_2H_4 production. C_2H_4 production was measured by the gas chromatographic assay method. The line through the experimental points corresponds to the best fit to the Michaelis-Menten equation. Other experimental details were as described in the text.

The average value for V_{max} for nitrogen fixation was 23 μmol/h per mg of chlorophyll, compared with the average value for V_{max} for C_2H_2 reduction of 82 μmol/h per mg of chlorophyll. This gives a conversion factor of 3.6.

Estimation of conversion factor by measurements of N_2 uptake and C_2H_2 reduction on the same batch of cyanobacteria. To obtain a more precise estimate of the conversion factor, we made comparative measurements of nitrogen uptake and acetylene reduction on the same batch of cyanobacteria. Since saturating could not be obtained for N_2 uptake, V_{max} for nitrogen uptake was measured as described above. Acetylene reduction was measured under saturating conditions (10% [vol/vol] C_2H_2).

The results from five batches of cyanobacteria are shown in Table 2. The conversion factor in this case was again greater than the stoichiometric value of 3. In fact, it was very close to 4, both for the experiment carried out at 35°C and for that carried out at 25°C.

Effect of C_2H_2 and N_2 on H_2 production. A possible contributory factor to the variation between the stoichiometric value of 3 for the conversion factor and the experimentally determined value of 4 is the production of H_2. It is well known that nitrogenase, in addition to reducing N_2 to NH_3, also reduces H^+ to H_2. We have previously reported that the reduction of protons to H_2 in *A. variabilis* is dependent on the concentration of dissolved N_2 (17). It is generally accepted that C_2H_2 is a better inhibitor of H^+ reduction than is N_2. To check whether this was the case for *A. variabilis* under our conditions, we measured the effect of the concentrations of

TABLE 2. Conversion factor between C_2H_2 reduction and N_2 uptake by *A. variabilis*

Batch[a]	V_{max} (μmol/h per mg of chlorophyll)		Ratio of C_2H_2 to N_2
	C_2H_2	N_2	
1	70	18	3.9
2	78	20	4.0
3	76	20	3.8
4	95	29	3.3
5 (25°C)	29	7.2	4.0

[a] Temperature was 35°C unless otherwise shown.

dissolved N_2 and C_2H_2 on the light-induced production of H_2. The result is shown in Fig. 4. To prevent reoxidation of H_2 by the cyanobacterial hydrogenase, this experiment was carried out with a gas phase without O_2 and in the presence of DCMU to inhibit photosynthetic oxygen evolution. In addition to showing the effect of the concentration of dissolved C_2H_2 and N_2 on H_2 production, Fig. 4 also shows the effect of the concentrations of N_2 and C_2H_2 on N_2 uptake and C_2H_2 reduction, respectively. H_2 production and either N_2 uptake or C_2H_2 reduction were measured by the mass spectrometer in the same experiment.

Increasing concentrations of both N_2 and C_2H_2 decreased the light-induced cyanobacterial H_2 production, but, whereas H_2 production was completely inhibited by sufficiently high concentrations of C_2H_2, it was always observed when N_2 was being reduced. The H_2 evolution at 580 μM N_2 (0.96 atmospheres) was 22% of the maximum H_2 production without N_2.

Effect of temperature on the rate of nitrogen uptake acetylene production, and hydrogen production. In purified nitrogenase, the distribution of electrons between N_2 reduction and H_2 production is known to be affected by several factors, including temperature, pH, the ratio between the two component proteins of nitrogenase, and the ATP concentration (for a review, see reference 21).

To determine whether temperature also affects the distribution of electrons between N_2 reduction and proton reduction within the nitrogenase complex in intact cyanobacteria and thereby changes the conversion factor between N_2 fixation and acetylene reduction, we measured the temperature dependence of the three types of reduction.

The rate of N_2 uptake is not saturated at N_2 concentrations corresponding to those in air. Estimates of V_{max} thus require a knowledge of the value of K_m. We measured this at 25°C and found a value of 60 μM. This value lies within the range measured at 35°C (Table 1). Values of V_{max} for N_2 fixation at different temperatures were thus estimated from measurements of N_2 uptake rates at one known concentration and this value of K_m. This allowed the temperature dependence to be determined on one batch of cyanobacteria. This would be impractical if the dependence of rate on N_2 concentration were determined at each temperature.

Ethylene production was measured by gas chromatography by using a saturating concentration of C_2H_2 (10% [vol/vol]).

Hydrogen production was measured by mass spectrometry as described above. To prevent reoxidation of H_2 by the uptake hydrogenase, hydrogen production was measured with argon in the gas phase and in the presence of 10 μM DCMU.

The effect of temperature on the three reactions is shown in Fig. 5. Between 20°C and the optimal temperature of 40°C, the curves for N_2 uptake and C_2H_2 reduction are superimposable

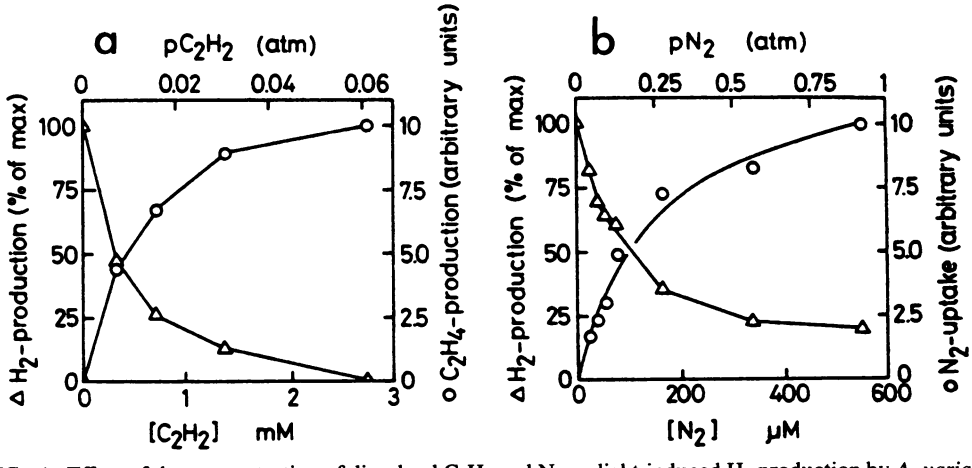

FIG. 4. Effect of the concentration of dissolved C_2H_2 and N_2 on light-induced H_2 production by *A. variabilis*. (a) Effect of the concentration of dissolved C_2H_2 on light-induced H_2 and C_2H_4 production. C_2H_4 and H_2 were measured by mass spectrometry in experiments similar to that shown for N_2 in Fig. 1. The gas phase contained argon and different concentrations of C_2H_2. H_2 was measured at $m/z = 2$, C_2H_2 at $m/z = 26$, and C_2H_4 at $m/z = 27$. DCMU (5 μM) was added to inhibit O_2 production. Measurements were made at 26°C. Other experimental details were as described in the text. (b) Effect of the concentration of dissolved N_2 on light-induced H_2 production and N_2 uptake. The experimental conditions were the same as in the experiment in (a), except that different concentrations of N_2 were used in place of C_2H_2.

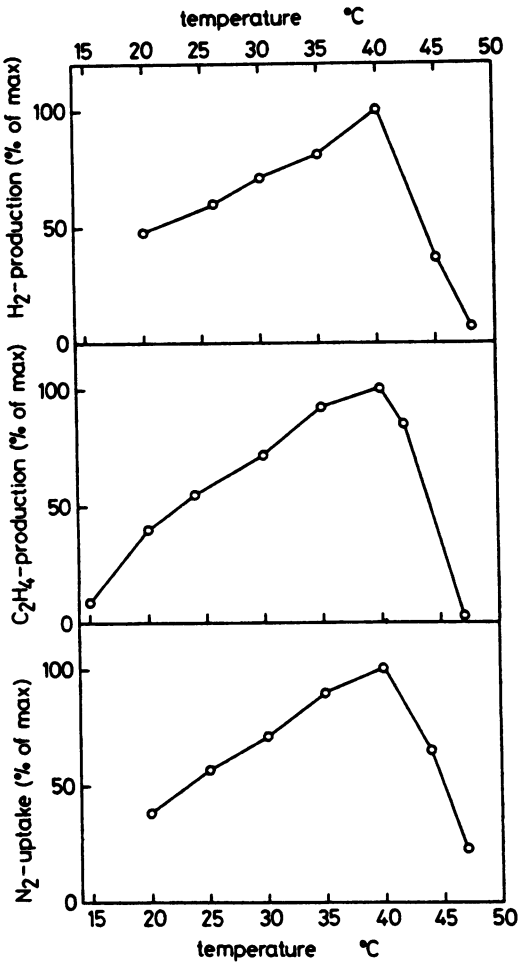

FIG. 5. Relative rates of light-induced C_2H_4 production, N_2 uptake, and H_2 production by *A. variabilis* as a function of temperature. The rates at 40°C were set to 100%. V_{max} for N_2 uptake was estimated by measuring the N_2 uptake rate with 25% N_2 in argon in the gas phase and calculating V_{max} from the results and the value previously found for K_m. To prevent reoxidation of H_2 by uptake hydrogenase, H_2 production was measured with 100% Ar in the gas phase and in the presence of 10 μM DCMU. Other experimental details were as described in the text.

and the temperature dependence of H_2 production is similar. The conversion factor between the two processes is thus apparently independent of temperature in this range. A replot of the results for C_2H_2 reduction as an Arrhenius plot showed two straight lines with an intersection at 21°C. Apparent activation energies were 36 and 166 kJ/mol above and below 21°C, respectively. Above 40°C, the rates decreased drastically with increasing temperature, although the rates observed were stable for at least the duration of the assay period (30 to 60 min).

DISCUSSION

Our measurements of the steady-state kinetics of N_2 uptake by intact *Anabaena* filaments give an average value for K_m of 65 μM (12.1 kPa). This means that the rate is still limited by N_2 concentration under normal conditions with 0.78 atmosphere of N_2 where it is only 90% saturated. The value for K_m is rather lower than the value we previously reported for the same organism in the presence of DCMU, which was 0.27 atmosphere of N_2 (17). We assume that this higher value is the result of the effect of the presence of H_2. This will be reoxidized by photosynthetically produced O_2 in the absence of DCMU but will accumulate in the presence of the inhibitor. Molecular hydrogen is known to act as a competitive inhibitor of N_2 reduction (4, 16, 24). We confirmed that N_2 fixation by *A. variabilis* was inhibited in the presence of H_2.

Our value for the K_m for N_2 in intact heterocystous cyanobacteria is close to the value of 0.11 atmosphere found by Stewart (26) for *Calothrix scrupulorum*. It is somewhat lower than the value of 0.20 atmosphere reported by Ohmori and Hattori (22) with *Anabaena cylindrica*, and considerably higher than the value of Burris and Wilson (5) for *Nostoc muscorum* of only 0.02 atmosphere. Our value also lies within the range reported for purified nitrogenase preparations from various diazotrophs (14).

Reported conversion factors between acetylene reduction and N_2 uptake for pure cultures of cyanobacteria and natural populations are compared in Table 3. The values range between 1.9 and 6.5, with many estimates lying close to 4. Average values for other types of nitrogen-fixing systems are 4.3 for heterotrophic bacteria, 4.3 for soil samples, 3.9 for symbiotic association with legumes, and 2.4 for nonlegumes (15). A value higher than 3.0 is expected as the result of the reduction of H^+ to H_2. This reaction is suppressed in the presence of sufficient C_2H_2, but even at saturating N_2 concentrations, 1 mol

TABLE 3. Reported values of the C_2H_2/N_2 conversion factor for heterocystous cyanobacteria

Organism	Conversion factor		Author and reference
	Range	Average	
A. cylindrica	2.4–4.1	2.8	Stewart et al. (28)
A. flos-aquae	3.0–3.2	3.2	
N. muscorum	3.3–4.0	3.6	
A. variabilis	3.3–4.0	3.8	This work
Cyanobacterial population	2.7–6.5	4.2	Peterson and Burris (23)
Cyanobacterial population		1.9	Burris (3)

of H_2 is produced for every mole of N_2 reduced. Three mechanisms have been proposed to explain H_2 production by nitrogenase (30). One of these involves obligatory production of H_2 by nitrogenase to yield a minimum H_2/N_2 ratio of 1. In this theory (6), H bound to a metal may act as a leaving group for the binding of N_2, i.e.,

$$M + 2H^+ + 2e^- \rightarrow M\text{—}\overset{H}{\overset{/}{}}H + N_2 \rightarrow MN + H_2$$

(If N_2 or other reducible substrate is absent, then protons can also react with these H atoms to give H_2.) This mechanism could give an overall reaction for N_2 reduction as

$$N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$$

that is, 1 mol of H_2 produced for each mole of N_2 reduced. This would give a theoretical C_2H_2/N_2 conversion factor of 4 and would in general agree well with our findings.

In any case, our results support the suggestion of Stewart (27) that in the absence of direct comparisons of C_2H_2 reduction with ^{15}N measurements or other methods of following N_2 fixation in the sample under investigation, a conversion factor of 4 is more appropriate than one of 3.

ACKNOWLEDGMENTS

We thank Martin Svendsen and Bodil Kristensen for their skilled technical assistance and Aase Reffstrup for being so helpful during the typing of the manuscript.

LITERATURE CITED

1. **Bergersen, F. J.** 1970. The quantitative relationship between nitrogen fixation and the acetylene-reduction assay. Aust. J. Biol. Sci. **23**:1015–1025.
2. **Booker, M. J., and A. E. Walsby.** 1979. The relative form resistance of straight and helical blue-green algal filaments. Br. Phycol. J. **14**:141–150.
3. **Burris, R. H.** 1976. Nitrogen fixation by blue-green algae of the Lizard Island area of the Great Barrier Reef. Aust. J. Plant. Physiol. **3**:41–51.
4. **Burris, R. H.** 1979. Inhibition, p. 569–604. *In* R. W. F. Hardy (ed.), A treatise on dinitrogen fixation, I and II. John Wiley & Sons, London.
5. **Burris, R. H., and P. W. Wilson.** 1946. Characteristics of the nitrogen-fixing enzyme in *Nostoc muscorum*. Bot. Gaz. **108**:254–262.
6. **Chatt, J.** 1980. Chemistry relevant to the biological fixation of nitrogen, p. 1–18. *In* W. D. P. Stewart and J. R. Gallon (ed.), Nitrogen fixation. Annual Proceedings of the Phytochemical Society of Europe, vol. 18. Academic Press, Inc., London.
7. **Chemical Rubber Company.** 1962. Handbook of Chemistry and Physics, 44th ed., p. 1706 and 1708. CRC Press, Cleveland, Ohio.
8. **Degn, H., J. S. Lundsgaard, L. C. Petersen, and A. Ormicki.** 1980. Polarographic measurement of steady state kinetics of oxygen uptake by biochemical samples. Methods Biochem. Anal. **26**:47–77.
9. **Degn, H., and H. Wohlrab.** 1971. Measurement of steady-state values of respiration rate and oxidation levels of respiratory pigments at low oxygen tensions. A new technique. Biochim. Biophys. Acta **245**:347–355.
10. **Dilworth, M. J.** 1966. Acetylene reduction by nitrogen-fixing preparations from *Clostridium pasteurianum*. Biochim. Biophys. Acta **127**:255–294.
11. **Duggleby, R. G.** 1981. A nonlinear regression program for small computers. Anal. Biochem. **110**:9–18.
12. **Falkenberg, P., B. Buckland, and A. E. Walsby.** 1972. Chemical composition of gas vesicles isolated from *Anabaena flos-aquae*. Arch. Microbiol. **85**:304–309.
13. **Fay, P.** 1980. Heterocyst isolation. Methods Enzymol. **69**:801–812.
14. **Hardy, R. W. F.** 1979. Reducible substrates of nitrogenase, p. 515–568. *In* R. W. F. Hardy, (ed.), A treatise on dinitrogen fixation, I and II. John Wiley & Sons, London.
15. **Hardy, R. W. F., R. C. Burns, and R. D. Holsten.** 1973. Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. Soil Biol. Biochem. **5**:47–81.
16. **Hwang, J. C., C. H. Chen, and R. H. Burris.** 1973. Inhibition of nitrogenase-catalyzed reduction. Biochim. Biophys. Acta **292**:256–270.
17. **Jensen, B. B., R. P. Cox, and H. Degn.** 1981. Mass spectrometric measurements of steady-state kinetics of cyanobacterial nitrogen fixation by monitoring dissolved N_2 in an open system. FEMS Microbiol. Lett. **12**:37–40.
18. **Lundsgaard, J. S., and H. Degn.** 1973. Digital regulation of gas flow rates and composition of gas mixtures. IEEE Trans. Biomed. Eng. **20**:384–387.
19. **Mackinney, G.** 1941. Absorption of light by chlorophyll solutions. J. Biol. Chem. **140**:315–322.
20. **Miller, S. A.** 1965. Acetylene, p. 74. Academic Press, Inc., New York.
21. **Mortenson, L. E., and R. N. F. Thorneley.** 1979. Structure and function of nitrogenase. Annu. Rev. Biochem. **48**:387–418.
22. **Ohmori, M., and A. Hattori.** 1972. Effect of nitrate on nitrogen-fixation by the blue-green alga *Anabaena cylindrica*. Plant Cell Physiol. **13**:589–599.
23. **Peterson, R. B., and R. H. Burris.** 1976. Conversion of acetylene reduction rates to nitrogen fixation rates in natural populations of blue-green algae. Anal. Biochem. **73**:404–410.
24. **Rivera-Oritz, J. M., and R. H. Burris.** 1975. Interactions among substrates and inhibitors of nitrogenase. J. Bacteriol. **123**:537–545.
25. **Schöllhorn, R., and R. H. Burris.** 1967. Acetylene as a competitive inhibitor of N_2 fixation. Proc. Natl. Acad. Sci. U.S.A. **58**:213–216.
26. **Stewart, W. D. P.** 1967. Nitrogen turnover in marine and brackish habitats. II. Use of ^{15}N measuring nitrogen fixation in the field. Ann. Bot. **31**:385–407.
27. **Stewart, W. D. P.** 1980. Systems involving blue-green algae (Cyanobacteria), p. 584–635. *In* F. J. Bergersen (ed.), Methods for evaluating biological nitrogen fixation. John Wiley & Sons, London.
28. **Stewart, W. D. P., G. P. Fitzgerald, and R. H. Burris.** 1968. Acetylene reduction by nitrogen-fixing blue-green algae. Arch. Microbiol. **62**:336–348.
29. **Tischer, G. R.** 1965. Pure culture of *Anabaena flos-aquae* A 37. Nature (London) **205**:419–420.
30. **Walker, C. C., C. D. P. Partridge, and M. G. Yates.** 1981. The effect of nutrient limitation on hydrogen production by nitrogenase in continuous cultures of *Azotobacter chroococcum*. J. Gen. Microbiol. **124**:317–327.